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Heat shock proteins (hsps) are induced in cells in response to environmental stresses. It has been shown that breast cancer cells sometimes express high levels of hsp27, which may augment the aggressiveness of these tumors and make them more resistant to treatment. The research funded by this fellowship is directed at regulating hsp27 toward the development of a potentially useful therapy for breast cancer.

This is the first year progress report on this project, and largely concerns specific aim 1, the examination of the regulatory mechanisms controlling the expression of hsp27 in breast cancer cells. We worked on two regions of the hsp27 promoter: a region largely responsible for basal promoter activity, which contained a 1/2ERE-TATA site, and a region containing a putative negative regulatory element. We cloned and purified the protein that binds the 1/2ERE-TATA site, and are currently investigating its impact on hsp27 expression. In addition, we began an analysis of the putative negative regulatory element by subcloning the region, and are testing these clones for regulatory activity.

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Suzanne Fuqua 10/25/96

Principal Investigator's Signature Date

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Abstract:

Heat shock proteins (hsps) are induced in cells in response to environmental stresses. It has been shown that breast cancer cells sometimes express high levels of hsp27, which may augment the aggressiveness of these tumors and make them more resistant to treatment. The research funded by this fellowship is directed at regulating hsp27 toward the development of a potentially useful therapy for breast cancer.

This is the first year progress report on this project, and largely concerns specific aim 1, the examination of the regulatory mechanisms controlling the expression of hsp27 in breast cancer cells. We worked on two regions of the hsp27 promoter: a region largely responsible for basal promoter activity, which contained a 1/2ERE-TATA site, and a region containing a putative negative regulatory element. We cloned and purified the protein that binds the 1/2ERE-TATA site, and are currently investigating its impact on hsp27 expression. In addition, we began an analysis of the putative negative regulatory element by subcloning the region, and are testing these clones for regulatory activity.

Introduction:

Heat shock proteins (hsps) are molecules whose expression is induced in eukaryotic cells by a variety of environmental stresses (1). The increased expression of hsps has been observed in cells and tissues representing a broad spectrum of human diseases, including some types of cancer (2). Hsp27 is often found at high levels in human breast cancer cells (3). Our research has shown that hsp27 is involved in the regulation of breast cancer cell growth as well as the development of drug resistance in these cells (4). Recently, we have also demonstrated a role for hsp27 in the motility and invasion of breast cancer cells (5). Since drug resistance and progression/proliferation continue to be major

problems in the treatment of breast cancer, an investigation of hsp27 might result in an effective therapy for the treatment/control of breast cancer.

This study was designed to determine the role of hsp27 in resistance to specific chemotherapeutic drugs, and to begin dissecting the regulation of hsp27 in human breast cancer cells. Toward this goal, we proposed the following specific aims:

1. Examine the regulatory mechanisms underlying the expression of hsp27 in breast cancer cell lines and human breast tumors. (Years 1-3)
2. Identify genes whose expression is associated with hsp27 effects on proliferation and drug resistance, as well as proteins interacting directly with hsp27. (Year 3)
3. Target positive and negative hsp27 transcriptional regulatory factors identified in Aims 1 and 2 to interfere with hsp27 expression. (Years 2-3)

The work addressing Aim 1, carried out in our laboratory between 30 January 1995 to 30 January 1996 is the subject of this report.

Body:

The first year of investigation was spent addressing specific aim 1. As discussed in the grant, we cloned the hsp27 promoter region and identified a broad area involved in basal transcription and a negative response region by deletion mapping (6). This report concerns the progress we have made working on these two regions of the hsp27 promoter.

We identified a putative transcription factor that binds to a unique sequence in a region of the hsp27 promoter that was responsible for the majority of basal promoter activity. At the time the grant was written, we had demonstrated the ability to partially

purify the protein. This year we continued our work on purifying and cloning this factor. We decided to call the protein HET, for hsp27-ERE-TATA binding protein, since it binds a 1/2ERE-TATA site in the hsp27 promoter. A partial cDNA clone of 0.9 kb was first isolated by screening an MCF-7 expression library with the hsp27 promoter fragment (-99 bp to -15 bp). The partial cDNA clone was then expressed and purified as a GST fusion protein. This protein specifically bound the hsp27 promoter fragment, whereas GST alone did not bind (Figure 1).

Next, we embarked on cloning the full length cDNA for HET. This proved to be a challenging task. Screening of three cDNA libraries (two MCF-7 breast cancer libraries from our laboratory and a brain library from Stratagene) resulted in clones that were identical in some regions and not in others. We were not sure whether this was because the protein is part of a large gene family, or because the libraries we were using were of poor quality. We then turned to RACE (rapid amplification of cDNA ends), using the Marathon cDNA Amplification Kit (Clontech). Again, we obtained clones that were only partially identical. Finally, however, screening of yet another MCF-7 breast cancer cDNA library (a kind gift from Dr. Jeff Marks, Duke University Medical Center, North Carolina) resulted in the identification of a longer clone of 1.6 kb, which overlapped with the first clone. Coupled in vitro transcription/translation of this clone yields a product of approximately 130 kDa (Figure 2), which agrees with the estimated size from Southwestern Blot shown in the original grant.

Periodic searching of GENEbank led to the discovery that 1.2 kb of 1.6 kb from our HET clone has 100% homology to a scaffold attachment factor, called SAF-B (7). SAF-B was cloned from a HeLa cell cDNA library. The remaining length of our clone (0.4

kb) shows less homology (55%) at the nucleotide level, although there is still high homology at the amino acid level (70%).

The homology between HET and SAF-B leads us to hypothesize that HET is a nuclear matrix protein. This is exciting because of the increasing realization over the past few years of the importance of higher order structure (e.g. chromatin structure, nucleosomes, and the nuclear matrix) in transcriptional regulation. The nuclear matrix is composed of a number of proteins which provide anchor sites for DNA attachment. These proteins can serve a variety of functions. They may be transcription factors that directly interact with DNA at promoter/enhancer/silencer sites, and/or may be part of the basal transcription machinery (8). They may be structural components which create the right "environment" for transcription (9). They may also indirectly regulate transcription by regulating other transcription factors (10).

We hypothesize that HET may affect hsp27 transcription by binding DNA and acting as a transcription factor. HET was originally identified as a factor that might regulate hsp27 transcription. It was cloned by screening an MCF-7 expression library with a fragment of the hsp27 promoter, indicating that HET binds DNA. Furthermore, the GST-HET fusion protein also bound the same promoter fragment in gel-shift assays. Therefore, the next step in our investigations of HET is outlined in specific aim 3, and scheduled for year 2. We plan to analyze the effect of HET on hsp27 transcription in transient as well as stable transfections. Stable transfections (which allow for interactions with the nuclear structure of the cell) are necessary, especially if this is a nuclear matrix protein. If HET alters the activity of hsp27 transcription, we will examine whether transfection of HET will alter the growth and/or drug resistance of breast cancer cells. The goal is to determine

whether increasing or decreasing HET to affect hsp27 expression has future therapeutic potential.

Specific aim 1 also involved the characterization of a region of the hsp27 promoter containing a putative negative regulatory element. We first tested whether the region could act as a negative element for heterologous promoters. The fragment was cloned in front of the hsp70 promoter and an SV40 promoter and tested in transient transfections (Figure 3). Our results indicate that it can indeed function with heterologous promoters. We have begun to deletion map the region by subcloning it in front of a heterologous promoter to be used in transient transfections. We are currently involved in this cloning process.

As indicated in the introduction of this report, specific aim 1 was scheduled for years 1-3. It involved an investigation of the regulatory mechanisms underlying the expression of hsp27 in breast cancer cell lines and human breast tumors. We have made significant progress toward that goal. Specific aim 3 was scheduled for years 2-3 and involved targeting positive and negative hsp27 transcriptional regulatory factors identified in Aims 1 to interfere with hsp27 expression. Again, we have already begun to analyze the effect of HET on hsp27 expression, so are on schedule. Finally, we will begin work on specific aim 2 at the end of year 2, to be completed in year 3.

Conclusion:

In the first year of this 3-year proposal, we have worked on understanding the regulation of hsp27 expression. We focused on two regions of the promoter: a region largely responsible for basal promoter activity, which contained a 1/2ERE-TATA site, and a region containing a putative negative regulatory element.

First, we cloned and purified the protein that bound the 1/2ERE-TATA site (HET). Sequence analysis indicates that it has high homology with another protein, SAF-B, which is a scaffold attachment factor. We plan to continue to study the role HET plays in the hsp27 regulation during the next year.

Second, we began to analyze the region containing the putative negative regulatory element. This element functions to decrease transcription from the hsp70 and SV40 promoters, as well as the hsp27 promoter. Further investigations will involve identification of the sequence responsible for this negative activity, cloning, and purification of the protein(s) bound to this sequence.

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Figure Legends:

Figure 1: GST-HET fusion protein binds to the hsp27 promoter. The left panel represents a Coomassie stained SDS-PAGE of extracts from E.coli expressing GST-HET fusion protein and GST only as a negative control, before and after purification on glutathione beads. The purified fractions (GST-HET and GST) were then used in a gel-retardation assay using the hsp27 promoter fragment as a probe. Only GST-HET, not GST, binds in a concentration-dependent (2 and 10 µl) manner.

Figure 2: In vitro transcription/translation of full-length HET clone. After in vivo excision with helper phage HET was recovered in a Bluescript plasmid, which could then be directly used for in vitro transcription/translation analysis. 2ug DNA were in vitro transcribed and translated using TnT coupled Reticulocyte Lysate system (Promega) in the presence (left panel) or absence (right panel) of ^{35}S-methionine. The products were then separated by SDS-PAGE, dried and exposed to a film (left panel), or transferred to nitrocellulose and incubated with 1:1000 dilution of HET-specific antibody (right panel). The signal was developed using ECL.

Figure 3: Activity of the putative negative regulatory element with heterologous promoters. Relative luciferase units are expressed as luciferase activity divided by beta-galactosidase activity of a cotransfected beta-galactosidase construct.

FIGURE 1: GST-HET fusion protein binds to the hsp27 promoter

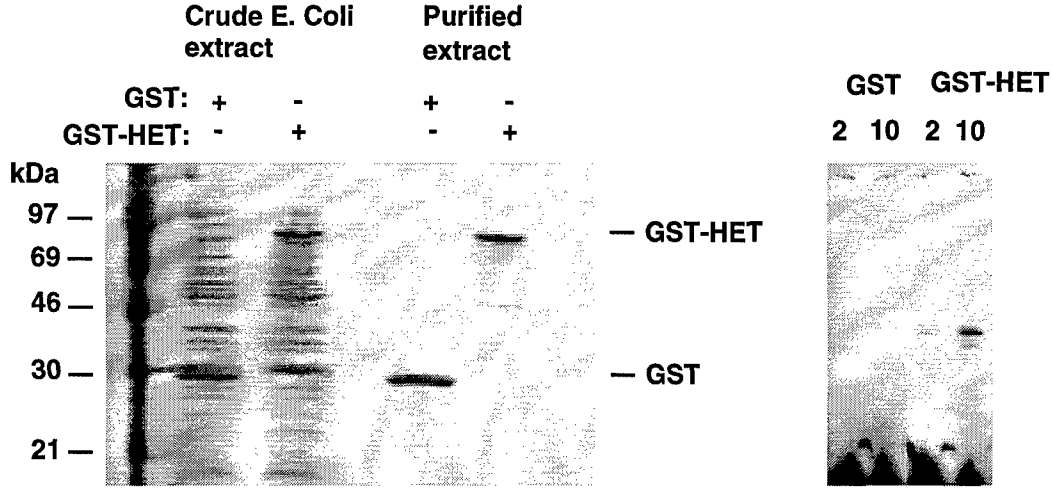


FIGURE 2: In vitro transcription/translation of full-length HET clone

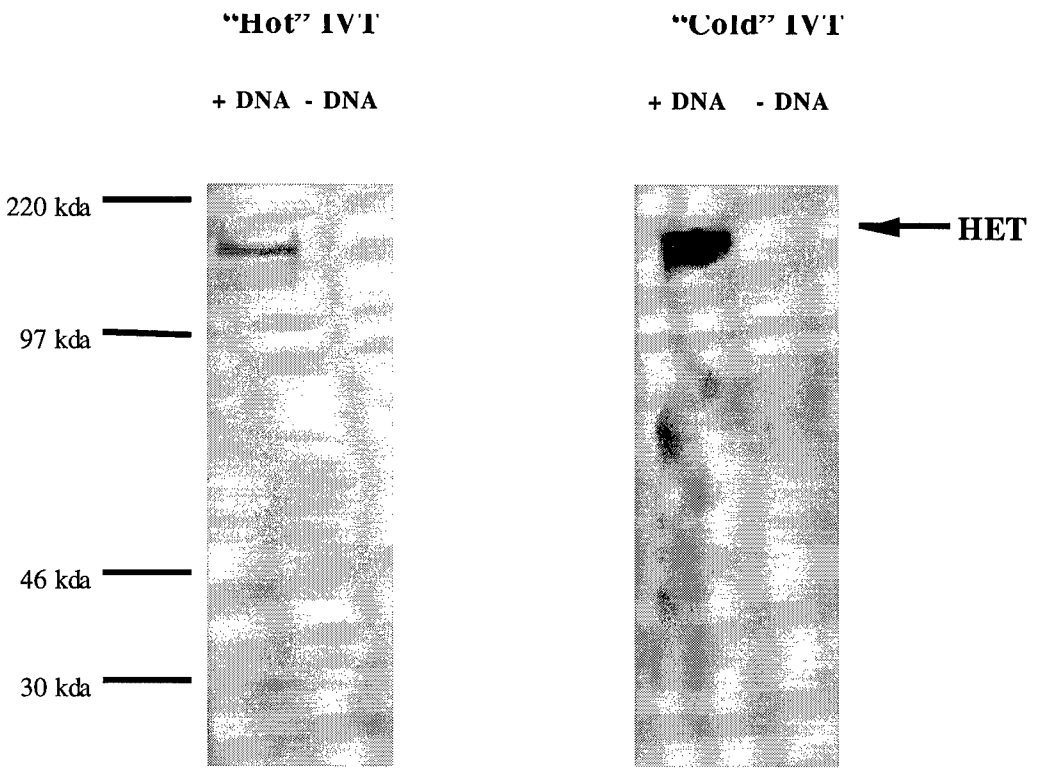
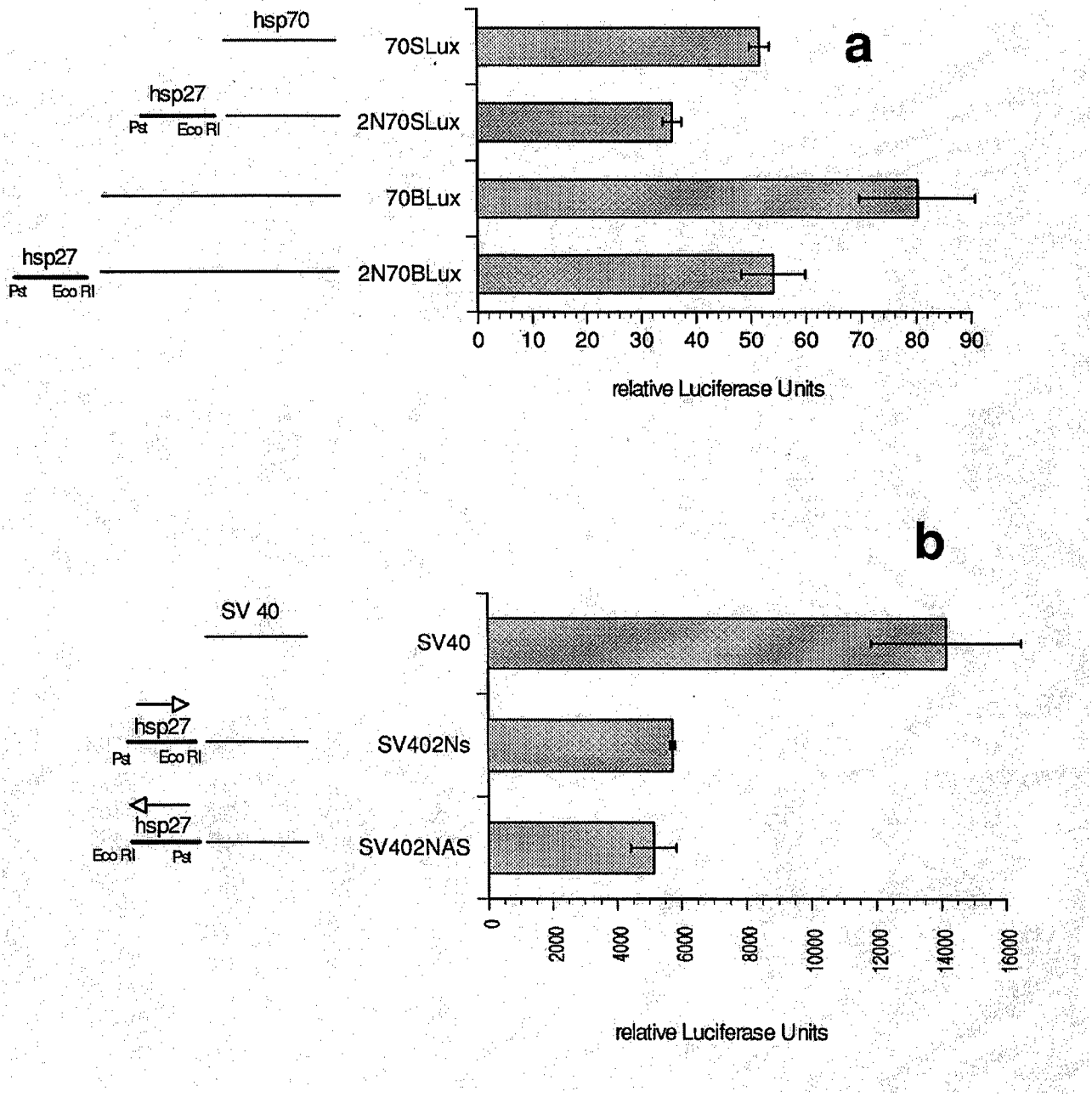


Fig. 3: Activity of the putative negative regulatory element with heterologous promoters



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